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The adaptation of *Rice yellow mottle virus* to the eIF(iso)4G-mediated rice resistance

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ABSTRACT

The *rymv1–3* allele of the eIF(iso)4G-mediated resistance to *Rice yellow mottle virus* (RYMV) is found in a few *Oryza glaberrima* cultivars. The same resistance-breaking (RB) mutations emerged in the central domain of the VPg after inoculation of isolates of different strains. The RB mutations were fixed, often sequentially, at codons 41 and 52 which paralleled an increase in virus accumulation. RB mutations also emerged after inoculation of an avirulent infectious clone, indicating that they were generated *de novo* in resistant plants. Only virus isolates with a threonine at codon 49 of the VPg broke *rymv1–3* resistance, those with a glutamic acid did not. A small subset of these isolates overcame *rymv1–2* resistance, but following a specific pathway. Comparison with the RB process of *rymv1–2*, a resistance allele found in a few *Oryza sativa* cultivars, showed similarities in the mode of adaptation but revealed converse virulence specificity of the isolates.

Introduction

Plant viruses are comparatively easy to handle and, consequently, are increasingly used to investigate virus adaptation to new hosts (Elena et al., 2008; Ohshima et al., 2010; Roossinck, 2008; Van der Walt et al., 2009). Studies of the breakdown of resistance, initially motivated by the search for durable resistances (Garcia-Arenal and McDonald, 2003; Gomez et al., 2009; Harrison, 2002), proved to be particularly useful in this respect because, in several instances, both virulence and resistance factors are identified. This is exemplified by recessive resistances mediated by eukaryotic translation initiation factors. Mutations in the VPg (viral protein, genome-linked) of several potyviruses confer the ability to overcome these resistances (Robaglia and Caranta, 2006). This is also true of *Rice yellow mottle virus* (RYMV), of the genus *Sobemovirus*, which causes a major rice disease in Africa (Kouassi et al., 2005). Only one gene for high resistance to RYMV, named *rymv1*, has been found in rice. This resistance is recessive and mediated by an eIF(iso)4G gene (Albar et al., 2006). Four resistance alleles have been identified, one in the Asiatic rice *Oryza sativa*, three in the African rice *Oyrza glaberrima* (Thiémélé et al., 2010). After inoculation of *rymv1*-resistant plants, irrespective of the resistance allele, the impact on yield is negligible, no symptoms are visible and the virus is not detected in ELISA tests (Ndjiondjop et al., 1999; N'Guessan et al., 2001). However, *rymv1* resistance is not expressed as immunity as RYMV was detected in *rymv1–2* resistant plants by quantitative PCR (Poulicard et al., 2010).

The resistance allele *rymv1–2*, identified in the Asiatic rice *O. sativa* cvs. Gigante and Bekarosaka, is due to a point mutation leading to the replacement of a glutamic acid by a lysine at codon 309 in the central domain of eIF(iso)4G (Albar et al., 2006; Rakotomalala et al., 2008). The adaptation of RYMV to the *rymv1–2* allele resistance in rice has been studied in detail (Fargette et al., 2002; Hébrard et al., 2006, 2008; Pinel-Galzi et al., 2007; Poulicard et al., 2010; Traoré et al., 2006). The *rymv1–2* resistance is most frequently overcome by the substitution at codon 48 of the VPg of an arginine by a glycine, and then a glycine by a glutamic acid. Another two-step mutational pathway at codon 48 is the substitution of an arginine by an isoleucine, and then an isoleucine by a valine. At codon 52, a histidine is replaced by a tyrosine.

The objective of this study is to distinguish the general from the allele-specific features of the *rymv1* resistance-breaking process. The analysis was then extended to the *rymv1–3* allele found in the cultivar Tog5681 and a few other *O. glaberrima* cultivars (Thottappilly and Rossel, 1993). Resistance of *rymv1–3* is caused by a deletion of codons 322–324 in the same domain of eIF(iso)4G (Albar et al., 2006). An approach similar to that followed for *rymv1–2* was adopted. A large range of isolates representative of the geographic distribution and of the genetic diversity of RYMV was inoculated to *rymv1–3*-resistant plants. Resistance-breaking mutations were determined by comparison of the sequences of the VPg before and after resistance-breakdown. Major resistance-breaking (RB) mutations were validated by mutagenesis of an infectious clone. The fixation of mutations over time was followed, the mutational pathways to *rymv1–3* virulence

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were reconstructed, and the virus multiplication of the mutants was assessed by quantitative PCR. The ability of an avirulent infectious clone to fix *rymv1–3* RB mutations after direct inoculation of resistant plants was also tested. Finally, a molecular signature of the differences among isolates in their ability to overcome *rymv1–3* resistance was sought in the VPg. Comparison of the *rymv1–2* and *rymv1–3* RB processes showed marked similarities in the mode of viral adaptation to *rymv1*. Yet it revealed opposite virulence specificity of the isolates as genotypes breaking *rymv1–3* resistance hardly broke *rymv1–2* and vice versa.

Results

The rymv1–3 RB mutations

Overall, *rymv1–3* resistance was overcome by 21 out of the 84 isolates tested (i.e. 25%) (Fig. 1). The 21 *rymv1–3* RB isolates originated exclusively in West Africa and belonged to three phylogenetic groups. Sixteen *rymv1–3* RB isolates came from the West of Niger and were collected in two independent surveys along the Niger River. They belonged to a variant of the S1-ca strain which is found exclusively in eastern countries of West Africa (Bénin, Niger, Togo). Three isolates were of the S2–S3 strain which is most prevalent in the forested part of West Africa. Two other *rymv1–3* RB isolates belonged to the S1 strain found in savannah zones of West Africa. Interestingly, only four of these 21 *rymv1–3* RB isolates could also overcome *rymv1–2*. Three came from the West of Niger and clustered in the S1-ca strain, whereas one of them originated from Mali (Ma203) and belonged to strain S2–S3.

A total of 50 mutants was obtained from the 21 *rymv1–3* RB isolates. Mutations associated with the RB phenotype occurred between codons 37 and 52 of the VPg, mostly at codons 41 and 52 (Fig. 2). Most mutations were transitions and resulted in amino-acids with biochemical properties different from those of the avirulent

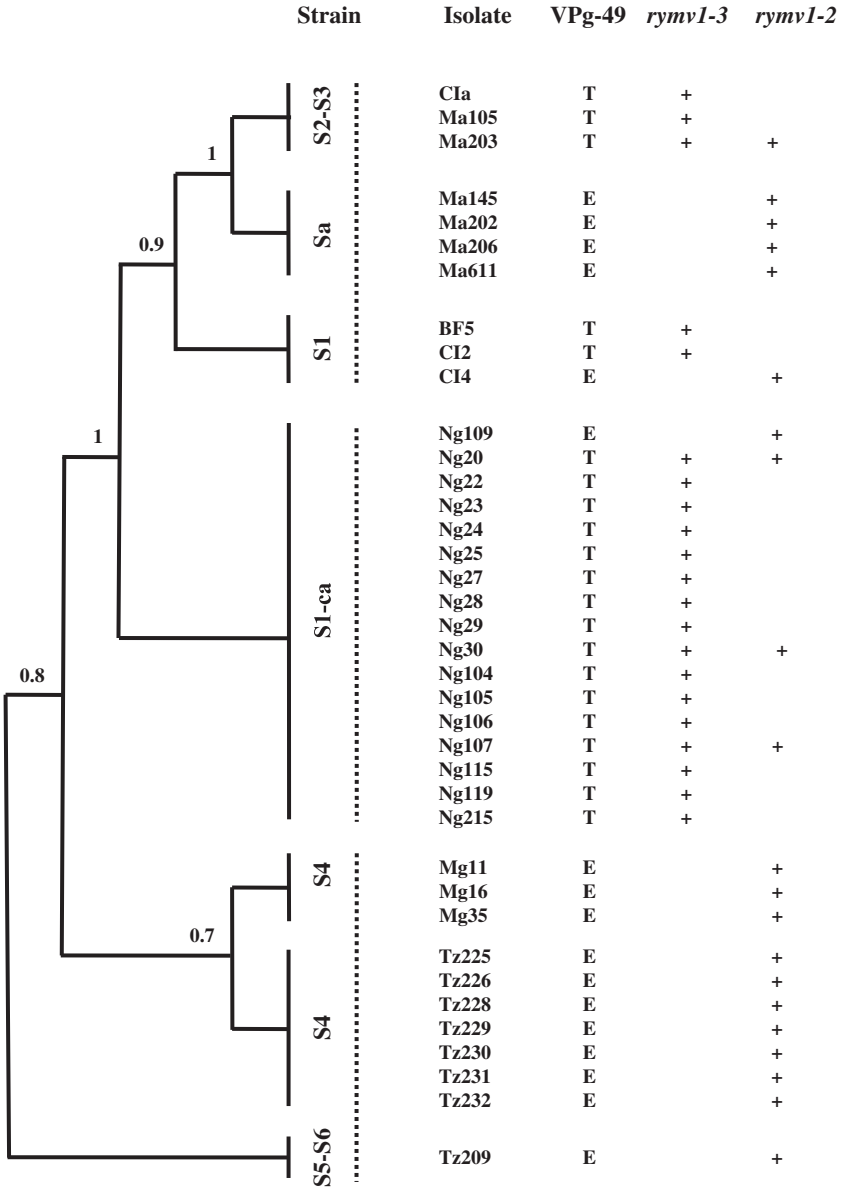

Fig. 1. Origin of the resistance-breaking isolates. The cladogram was reconstructed from the full sequence of reference isolates by maximum likelihood methods with bootstrap support of their nodes indicated. Each isolate was assigned to a particular strain after phylogenetic analysis of its coat protein gene. The strain and the geographic origin are indicated: strains S1, S2–S3 and Sa (West Africa), strain S1-ca (Central Africa and neighboring countries of West Africa), strains S4 and S5–S6 (East Africa). The amino acid at codon 49 of the VPg is abbreviated by an 'E' for glutamic acid and by a 'T' for threonine. Resistance-breakdown of *rymv1–3* and *rymv1–2* is marked by a '+'. Resistance-breakdown of *rymv1–2* was first established in earlier studies with a lower number of isolates (Pinel-Galzi et al., 2007).

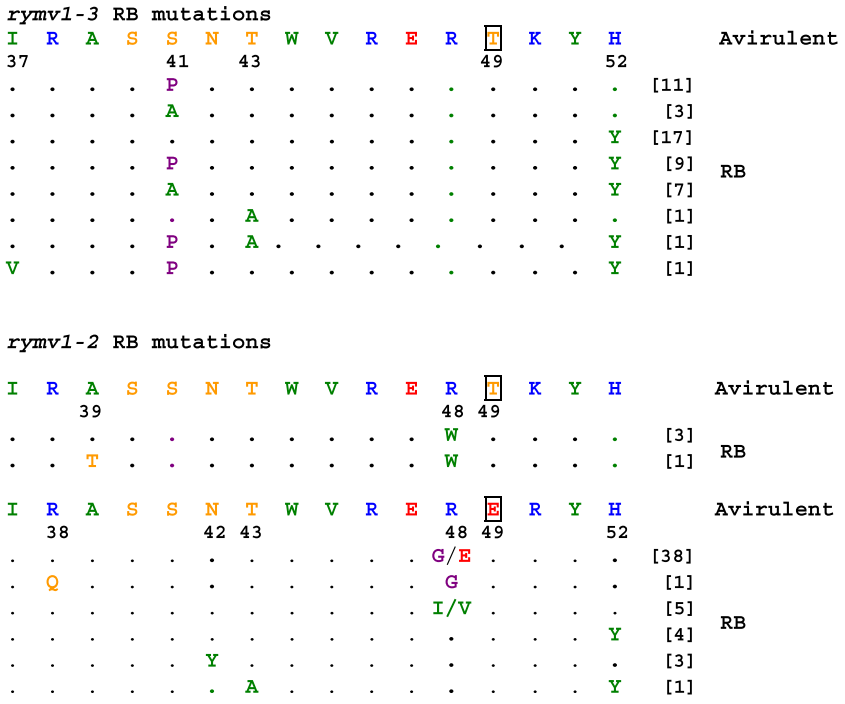

Fig. 2. Resistance-breaking mutations. Amino-acid changes associated with *rymv1–3* and *rymv1–2* resistance-breakdown were deduced from comparison of the VPg sequences of the wild avirulent and of the evolved forms of each isolate. The sequences of the avirulent-type are at the top lines. The isolates with a threonine and a glutamic acid at codon 49 are distinguished (see text for explanation). The class of the amino-acids is indicated with colors: acidic (red), basic (blue), hydrophobic (green), polar (orange), and small (purple). For each amino acid change (or combination of changes), the number of RB mutants is shown in brackets at the right of the sequence. The *rymv1–2* RB mutations were first established in earlier studies with a lower number of mutants (Pinel-Galzi et al., 2007).

isolates. Both codons 41 and 52 were monomorphic in avirulent isolates. Moreover, codon 41 was under a conservative selection pressure with a significant *P* value ($P=9\times10^{-7}$). At codon 52, a histidine was substituted by a tyrosine. At codon 41, serine was most often substituted by a proline and sometimes by an alanine. The amino-acid replacement at position 41 was not dependent on codon usage. Proline derived from serine which was coded by TCC and TCA; alanine derived from serine which was coded by TCC, TCG and TCT (Fig. 3). The amino-acid replacement was not strain-dependent either, as isolates of S1-ca variant from West Niger broke resistance by fixation of a proline or an alanine. The causal role of mutations 52Y, 41P and 41A in *rymv1–3* resistance breakdown was validated by directed mutagenesis of an infectious clone. The resistance was readily overcome after inoculation of the transcripts Mutations at each of position 41 or 52 in the VPg induced resistance breakdown. The infectious clone mutated at these two positions also broke the *rymv1–3* resistance.

The four isolates which broke both resistance alleles overcame *rymv1–3* resistance by following the mutational pathways of any other *rymv1–3* RB isolate, a proline at codon 41 and/or a tyrosine at codon 52. By contrast, they broke *rymv1–2* resistance by a specific substitution; the replacement of the arginine by a tryptophan at codon 48 (Fig. 2). Interestingly, when mutation 48W was introduced into the infectious clone of isolate CIa, the mutated clone infected neither resistant nor susceptible plants. Then, mutation 48W fixed in three isolates of strain S1-ca and in one isolate of the S2–S3 strain (isolate Ma203) was lethal in the infectious clone CIa of the S2–S3 strain.

The rymv1–3 RB mutational pathways

The *rymv1–3* resistance was overcome after direct inoculation of transcripts of the avirulent infectious clone; systemic symptoms developed and the mutation 41P was fixed. This is the first report of adaptation of an infectious clone to the *rymv1* resistance. In avirulent isolates, RB mutations at codons 41 and 52 emerged at a similar frequency. A first mutation either at codon 41 or 52—there was no order of appearance—was often followed by the fixation of the other one, as revealed when following the course of infection (Fig. 3). Consistently, mutation 52Y was fixed after inoculation of the infectious clone mutated with 41P. In no instance was a mutant displaced by the alternate one during the *rymv1–3* RB process.

The quantitative RT-PCR technique (qRT-PCR) was applied to assess the viral accumulation in *rymv1–3* resistant plants after inoculation of the avirulent isolate, of the single RB mutants, and of the double RB mutants. The following trends were apparent (Fig. 4). After inoculation of the avirulent isolate, the sensitive qRT-PCR technique detected a low but consistent viral RNA accumulation, above the threshold level set with test of non-inoculated leaves. When a single RB mutation was fixed, be it 52Y, 41P or 41A, the virus accumulation of the single mutant was significantly higher than the avirulent isolate ($P=0.003$ in one-sided Student test with unequal variance), i.e. 10 to 10^2-fold greater than the WT. When both mutations were fixed together, be it 41P and 52Y or 41A and 52Y, the virus accumulation of the double mutants was significantly higher than the single mutants ($P=0.002$ in one-sided Student test with unequal variance), i.e. c. 10^5 greater than the avirulent isolate. After this stepwise RB accumulation, the virus content in resistant plants reached c. 10^{12} viral RNA copies per milligram of leaf.

The ability to break the rymv1–3 resistance

The 21 isolates which broke *rymv1–3* resistance had a threonine at codon 49 of the VPg (Fig. 1, Table 1). By contrast, none of the 49 isolates with a glutamic acid at this position became virulent when inoculated to *rymv1–3*. Increasing the number of plants challenged

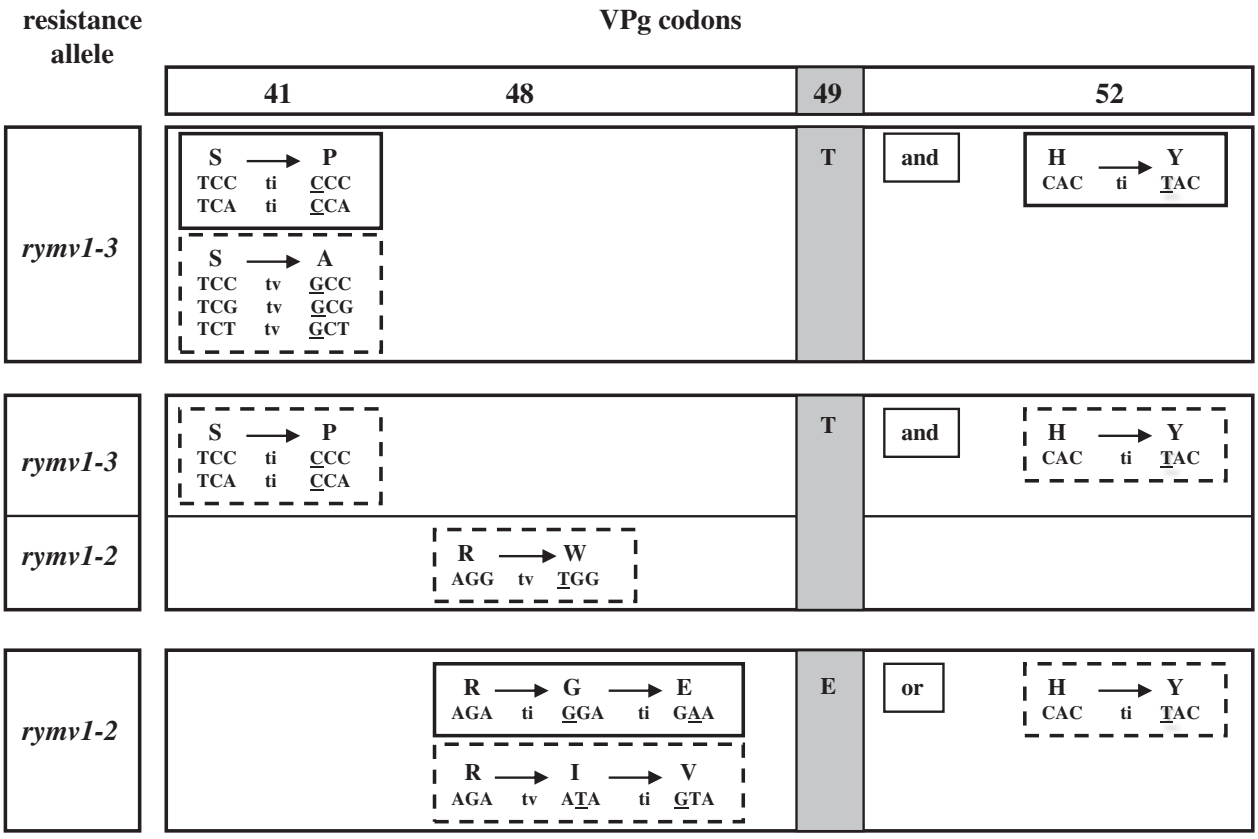

Fig. 3. Resistance-breaking mutational pathways. The mutational pathways to virulence against *rymv1–3* and *rymv1–2* are illustrated. The amino-acid and the nucleotide changes, and the type of nucleotide substitution are shown (ti for transition and tv for transversion). Plain and broken boxes symbolize frequent and rare mutations, respectively. The boxed words 'and' vs. 'or' indicate accumulation and displacement of RB mutations, respectively. The amino acid at codon 49 of the VPg is abbreviated by an 'E' for glutamic acid and by a 'T' for threonine and shaded. The *rymv1–2* mutational pathways were first established in earlier studies with a lower number of mutants (Pinel-Galzi et al., 2007).

with isolates with a glutamic acid did not result in a successful infection. The link between the ability to break *rymv1–3* resistance and the amino-acid at codon 49 was highly significant ($\chi^2 = 37.9$, $P<0.001$).

There was a converse relationship between the ability to break *rymv1–2* resistance and the amino-acid at codon 49 ($\chi^2 = 18.9$, $P<0.001$). Seventeen of the 21 *rymv1–2* RB isolates had a glutamic acid at this position. The other four were isolates with a threonine (Fig. 2). They overcame *rymv1–2* by fixation of a specific mutation, a tryptophan at codon 48. They were the only isolates that broke both *rymv1–2* and *rymv1–3* resistances.

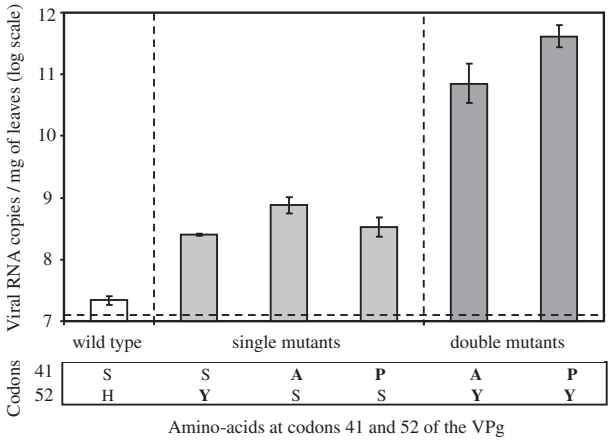

Fig. 4. Viral accumulation of RB mutants. Viral accumulation in *rymv1–3* resistant plants of the avirulent isolate CIa (41S, 52H in the VPg), of the single mutants (52Y, 41A, 41P) and of the double mutants (41A and 52Y, 41P and 52Y). The amino acids of the avirulent isolate are in plain letters, the mutated codons in bold letters. The number of viral RNA copies per milligram of fresh leaf was estimated by qRT-PCR at 30 days post-inoculation. Three plants were inoculated with each viral genotype. The vertical bars show the standard error of the means. The broken lines indicate the detection threshold.

Discussion

The adaptation process of RYMV to *rymv1–2* and *rymv1–3* resistance alleles showed striking similarities despite different genetic determinants (point mutation vs. deletion) and host species (*O. sativa* vs. *O. glaberrima*). RB mutations were never found in field isolates. Most *rymv1–3* RB mutations occurred between codons 37 and 52 in the central domain of the VPg. The substitutions were most often transitions, always coding, and resulted in amino-acids with biochemical properties different from those of the avirulent isolates. These results are consistent with those obtained with *rymv1–2* (Pinel-Galzi et al., 2007).

Table 1
Response of rice cultivars with rymv1–3 or rymv1–2 alleles of resistance to inoculation of RYMV isolates representative of the different strains in Africa with either a glutamic acid (E) or a threonine (T) at codon 49 of the VPg.

Viral isolates	Allele of resistance[a]			
aa at codon 49	rymv1–3		rymv1–2	
	−	+	−	+
E	49	0	40	17
T	14	21	88	4

[a] A '+' indicates resistance-breakdown.

The phylogeny of RYMV was initially based on the coat protein gene, but trees inferred from the other ORFs of the genome (including ORF2a which contain the VPg gene) and from the full genome are congruent (Pinel-Galzi et al., 2009). So the same *rymv1*–3 RB mutations were fixed whatever the phylogenetic origin of the isolates, a trait also observed with *rymv1–2*. It showed that parallel evolution is a general feature of *rymv1* resistance breakdown. It indicates that the number of mutational pathways for adaptation to *rymv1* resistance is low. It likely reflects the high specificity of the interactions between the VPg and eIF(iso)4G domains.

Both resistance alleles *rymv1–3* in *O. glaberrima* and *rymv1–2* in *O. sativa* were overcome by the H52Y substitution. A single amino-acid, at codon 303, close to the resistance mutations and deletions, differentiates *O. sativa* from *O. glaberrima* in the central domain of eIF(iso)4G (alanine vs. aspartic acid, respectively) (Albar et al., 2006; Thiémélé et al., 2010; C. Lirette and E. Hébrard, unpublished results). Then, H52Y substitution in the VPg of RYMV restored a compatible interaction with *rymv1–2* (309 K) in *O. sativa* (303A) and with *rymv1–3* (deletion of codons 322–324) in *O. glaberrima* (303D) in the central domain of eIF(iso)4G. The *rymv1–3* resistance-breakdown often involved a sequential accumulation of RB mutations at codons 41 and 52 which paralleled an increase in the accumulation rate. An amount of 10^{12} viral RNA copies per milligram of leaves was reached. A similar amount was estimated after resistance breakdown of *rymv1–2*, and in susceptible cultivars infected by WT isolates. Sequential fixation of RB mutations was also observed with *rymv1–2* (Pinel-Galzi et al., 2007). However, the stepwise breakdown process in *rymv1–2* progressed by the ordered replacement of RB mutations at the single codon 48, whereas with *rymv1–3* it consisted of the unordered accumulation of RB mutations at codons 41 and 52.

RB isolates are often assumed to pre-exit in the virus population prior to inoculation of the resistant plants. Alternatively, with RYMV, the stepwise mutational pathways (Pinel-Galzi et al., 2007) and the residual multiplication of the avirulent isolates on *rymv1–2*-resistant plants (Poulicard et al., 2010) suggested that RB mutations were generated *de novo* in resistant plants. Similarly, the *rymv1–3* RB process was characterized by a sequential accumulation of mutations at codons 41 and 52 and a residual multiplication of avirulent isolates in resistant plants. However, the only direct evidence of the production of RB variants during multiplication of avirulent isolates in resistant plants was the fixation of mutation 41P, a typical *rymv1–3* RB mutation, after inoculation of an avirulent infectious clone to *rymv1–3*-resistant plants. This validated the hypothesis that *rymv1–3* RB mutations are generated *de novo* in resistant plants rather than selected in susceptible plants from viral quasispecies.

Comparison of the *rymv1–2* and *rymv1–3* RB processes showed striking similarities which suggest a common mode of viral adaptation of RYMV to *rymv1* resistance. However, they also revealed converse virulence specificity of the isolates. No isolates from East Africa and hardly any from the savannah part of West Africa overcame *rymv1–3* resistance. Inversely, very few isolates from the forested part of West Africa broke *rymv1–2* resistance (Pinel-Galzi et al., 2007). The amino-acid at codon 49 of the VPg was a molecular signature of this converse virulence specificity. Strains with a glutamic acid at this position did not overcome *rymv1–3* whereas those with a threonine hardly broke *rymv1–2*. The exception was the small subset of isolates, mostly from West Niger, with a threonine at codon 49 of the VPg which overcome both alleles of resistance. They differed from any other isolates in breaking *rymv1–2* by the fixation of a tryptophan at codon 48. They also differed from the infectious clone CIa, and possibly from other isolates with a threonine at codon 49 unable to overcome *rymv1–2*, for which tryptophan at codon 48 was lethal.

Overall, the conditional emergence of RB mutation on the E/T polymorphism at codon 49 was the most salient difference in the adaptation processes to *rymv1–2* and *rymv1–3* resistances. Codon 49 is the only site of the VPg under positive selection (Pinel-Galzi et al., 2007), indicating that changes between a glutamic acid and a threonine at this position conferred a selective advantage during RYMV evolution. There is a striking correspondence between the geographical distribution of the two *Oryza* species and that of RYMV isolates. *O. sativa* is grown all over Africa, whereas *O. glaberrima* has been cultivated in West Africa only (Porterès, 1970; Semon et al., 2005; Thiémélé et al., 2010). Similarly, virus isolates with a glutamic acid at codon 49 of the VPg are spread all over Africa, whereas those with a threonine are restricted to West Africa (A. Pinel-Galzi, O. Traoré, D. Fargette, unpublished results). Our working hypothesis is that the amino acid at codon 49 reflects past adaptations to rice species and modulates the present ability to overcome the *rymv1* resistance alleles. Under this hypothesis, the isolates with a glutamic acid cannot overcome the *rymv1–3* resistance present in *O. glaberrima* cultivars because they are preferentially adapted to *O. sativa*. Conversely, the isolates with a threonine hardly break the *rymv1–2* resistance present in *O. sativa* because they are preferentially adapted to *O. glaberrima*. This would illustrate the principle that limitations upon present possibilities are consequences of past adaptations (Gould, 2002).

Materials and methods

Plant material and virus isolates

The cv. Tog5681 used in the experiments is one of the few *O. glaberrima* cultivars with the *rymv1–3* resistance. The plants were kept in a growth chamber under a 12-h illumination at 120 $\mu E/m^2/s$ at 28 °C and 60% humidity. Isolates representative of the strains of RYMV and originating from most rice-growing regions of Africa were collected from susceptible plants in the fields. They displayed a high genetic diversity with up to 17% uncorrected pairwise nucleotide differences in the coat protein gene. Strains S1 (savannah region), S2/S3 (mainly forest), and Sa (sahelian regions) originated from West Africa. Strain S1-ca was found in Central Africa and in neighboring countries of West Africa and accordingly referred to as the West-Central African strain. Strain S4 originated from continental East Africa and Madagascar, and S5/S6 exclusively from continental East Africa. Altogether, 84 isolates were inoculated to *rymv1–3*-resistant cultivars. A total of 50 RB genotypes was obtained from 21 RB isolates. Complementarily, the number of *rymv1–2* RB mutants was increased—from 41 derived from 10 isolates (Pinel-Galzi et al., 2007) to 56 from 21 isolates—in search of isolates able to break both alleles of resistance.

Inoculum was prepared by grinding infected frozen leaves in 0.1 M phosphate buffer (pH 7.2) (0.1 g/ml). Extracts were mixed with 600-mesh carborundum and rubbed on leaves of 11-day-old rice seedlings. In each experiment, the isolate was inoculated to 10–20 plants. Larger scale experiments with 50 plants were also conducted to obtain several RB mutants from the same isolate or to generate a RB mutant from a recalcitrant strain (Table S1). In several instances, samples were collected up to 6 months after inoculation of resistant plants to follow the course of fixation of RB mutations. Such a length of time is biologically realistic even for annually cultivated rice having a growing season of c. 3 months, as regrowth of infected rice stubble occurs frequently after harvest.

Sequencing and qRT-PCR tests

Isolates that induced generalized symptoms in resistant plants and/or high virus content as estimated by ELISA were collected. The VPg and the 5′ and 3′ neighboring regions were sequenced and compared to the sequence before inoculation. For phylogenetic purposes, the coat protein gene of the isolates was also sequenced. The cladogram derived from the phylogenetic tree which was reconstructed by maximum likelihood methods from the full sequence

of reference isolates (Pinel-Galzi et al., 2009). The VPg and CP genes and the full genome were sequenced as reported earlier (Fargette et al., 2004). Selection pressure expressed on codons involved in *rymv1–3* resistance breakdown was estimated on a corpus of 150 VPg sequences of isolates representative of the genetic diversity of RYMV and calculated as reported in Pinel-Galzi et al. (2007). Directed mutagenesis of the infectious clone CIa was performed as described elsewhere (Brugidou et al., 1995; Hébrard et al., 2006). Transcripts of the non-mutated infectious clone were also used to challenge plants having *rymv1–3* resistance. To assess the virus accumulation of the RB mutants on resistant plants, qRT-PRC assays were performed on plants that had been inoculated with c. 10^{11} copies of each viral genotype. Three plants were inoculated with each viral genotype. The last expanded leaf of each plant was collected 30 days after inoculation. The total RNA from 0.05 g of leaves was purified (RNeasy Plant Mini Kit, Quiagen, Hilden, Germany). Two replicates of a two-step reverse transcription of a region overlapping the VPg cistron were performed for each RNA extract under the conditions described in Poulicard et al. (2010). All reactions were performed in duplicate, including the negative controls.

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2010.09.007.

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